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A novel mitogenic cyclin and uses thereof

The present invention relates to DNA sequences encoding mitogenic cyclins as well as to methods for obtaining the same. The present invention also provides vectors comprising said DNA sequences, wherein the DNA sequences are operatively linked to regulatory elements allowing expression in prokaryotic and/or eukaryotic host cells. In addition, the present invention relates to the proteins encoded by said DNA sequences, antibodies to said proteins and methods for their production. The present invention also relates to a method for controlling or altering growth characteristics of a plant and/or a plant cell comprising introduction and/or expression of one or more mitogenic cyclins functional in a plant or parts thereof and/or one or more DNA sequences encoding such proteins. The present invention further relates to diagnostic compositions comprising the aforementioned DNA sequences, proteins and antibodies. The present invention also relates to methods for the identification of compounds being capable of activating or inhibiting the cell cycle. Furthermore, the present invention relates to transgenic plant cells, plant tissue and plants containing the above-described DNA sequences and vectors as well as to the use of the aforementioned DNA sequences, vectors, proteins, antibodies and/or compounds identified by the method of the invention in plant cell and tissue culture, plant breeding and/or agriculture.

The control of cell cycle progression in eukaryotes is mainly exerted at two transition points: one in late G_1, before DNA synthesis, and one at the G_2/M boundary. Progression through these control points is mediated by cyclin-dependent protein kinase (CDK) complexes, which contain a catalytic subunit of approximately 34-kDa encoded by the *CDK* genes. CDK protein is active as a protein kinase only when it is bound to a second protein called cyclin. Both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* only utilise one *CDK* gene for the regulation of their

cell cycle. The kinase activity of their gene products $p34^{cdc2}$ and $p34^{cdc28}$ in *Sch. pombe* and in *S. cerevisiae*, respectively, is dependent on the regulatory proteins, so-called cyclins as mentioned above. Progression through the different cell cycle phases is achieved by the sequential association of $p34^{cdc2/cdc28}$ with different cyclins. Although in higher eukaryotes this regulation mechanism is conserved, the situation is more complex since they have evolved to use multiple CDKs to regulate the different stages of the cell cycle. In mammals, seven CDKs have been described defined as CDK1 to CDK7, each binding a specific subset of cyclins.

The activity of cdk/cyclin complexes is regulated at five levels: (i) transcription of the *CDK* and cyclin genes; (ii) association of specific CDKs with their specific cyclin partner; (iii) phosphorylation/ dephosphorylation of the CDK and cyclin subunits; (iv) interaction with other regulatory proteins such as SUC1/CKS1 homologues and cell cycle kinase inhibitors (CKI); and (v) cell cycle phase-dependent destruction of the cyclins.

In *Arabidopsis thaliana*, thusfar two *CDK* genes have been isolated, *CDC2aAt* and *CDC2bAt*, of which the gene products share 56% amino acid identity. Both CDKs are distinguished by several features. First, only *CDC2aAt* is able to complement yeast $p34^{cdc2/cdc28}$ mutants. Second, CDC2aAt and CDC2bAt bear different cyclin-binding motifs (PSTAIRE and PPTALRE, respectively), suggesting they may bind distinct types of cyclins. Third, although both *CDC2aAt* and *CDC2bAt* show the same spatial expression pattern, they exhibit a different cell cycle phase-specific regulation. The *CDC2aAt* gene is expressed constitutively throughout the whole cell cycle. In contrast, *CDC2bAt* mRNA levels oscillate, being most abundant during the S and G_2 phases.

In addition, multiple cyclins have been isolated from *Arabidopsis*. The majority displays the strongest sequence similarity with the animal A- or B-type class of cyclins, but also D-type cyclins have been identified. D-type cyclins are only distantly related to other cyclins. In mammals D-type cyclins act as growth sensors, with their expression depending more on extracellular cues than on the cell's position in the cell cycle. Consequently D-type cyclins has been suggested to mediate mitogenic stimuli with the release from quiescence. This is believed to be regulated through the hyperphosphorylation of the retinoblastoma protein (Rb) by CDK/cyclin D complexes.

Rb is a tumour suppressor protein which plays an important role in controlling the onset of cell division. In its hypophosphorylated form, Rb is complexed with E2F-type transcription factors which are known to promote expression of S phase-specific genes. Binding of Rb to E2Fs thereby prevents S phase induction. Phosphorylated Rb is unable to form complexes with E2F transcription factors and allows DNA synthesis. All D-type cyclins show a specific amino acid motif (LXCXE) permitting them to bind Rb. In *Arabidopsis thaliana* three D-type cyclins have been identified by their ability to rescue a mutant yeast strain deficient in its G1-specific cyclins (Soni, Plant Cell 7 (1995), 85-103). These D-type cyclins only show about 30% amino acid identity to each other and were demonstrated to be differentially expressed: *CYCD1;1* is most abundant in flowers and leaves, *CYCD2;1* in leaves and roots, and *CYCD3;1* in roots. By the use of synchronized *A. thaliana* suspension cultures, achieved by the release from a block in late G1, it was shown that *CYCD3;1* transcripts increase at the G1/S transition point and remain constant thereafter. The *CYCD2;1* transcript level remains unchanged during the whole cell cycle (Fuerst, Plant Physiol. 112 (1996), 1023-1033). Therefore, the *A. thaliana* D-type cyclin genes are expressed in a cell cycle phase-independent manner, just as described for their mammalian counterparts. Rather, transcription is regulated by the presence of mitogens. In starved cell cultures, *CYCD2;1* expression is inducible by the addition of sucrose only, whereas *CYCD3;1* transcription is inducible by cytokinins (Soni, Plant Cell 7 (1995), 85-103). The observation that distinct D-type cyclins respond to different mitogenic stimuli suggests that each of them is involved in particular signal transduction pathways linking the perception of mitogenic stimuli with the cell cycle.

In order to manage problems related to plant growth, plant architecture and/or plant diseases, it is believed to be of utmost importance to identify and isolate plant genes and gene products involved in the regulation of the plant cell division. If such novel genes and/or proteins have been isolated and analyzed, the growth of the plant as a whole can be influenced. Also, the growth of specific tissues or organs and thus the architecture of the plant can be modified.

Thus, the technical problem underlying the present invention is to provide means and methods for modulating cell cycle proteins that are particular useful in agriculture and plant cell and tissue culture.

The solution to the technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the invention relates to a DNA sequence encoding a mitogenic cyclin or encoding an immunologically active and/or functional fragment of such a protein, selected from the group consisting of:

(a) DNA sequences comprising a nucleotide sequence encoding a protein comprising the amino acid sequence as given in SEQ ID NO: 2;
(b) DNA sequences comprising a nucleotide sequence as given in SEQ ID NO: 1;
(c) DNA sequences hybridizing with the complementary strand of a DNA sequence as defined in (a) or (b);
(d) DNA sequences encoding an amino acid sequence which is at least 70% identical to the amino acid sequence encoded by the DNA sequence of (a) or (b);
(e) DNA sequences, the nucleotide sequence of which is degenerated as a result of the genetic code to a nucleotide sequence of a DNA sequence as defined in any one of (a) to (d); and
(f) DNA sequences encoding a fragment of a protein encoded by a DNA sequence of any one of (a) to (e).

The term "mitogenic" refers to compounds (chemicals or proteins) which positively influence reentry into the cell cycle and/or progression of the cell cycle; see also Example 4. The term "Cyclin" means one of the proteins which actively regulate cell division.

The term "cell cycle" means the cyclic biochemical and structural events associated with growth of cells, and in particular with the regulation of the replication of DNA and mitosis. The cycle is divided into periods called: G_0, Gap_1 (G_1), DNA synthesis (S), Gap_2 (G_2), and mitosis (M).

The term "proliferation" means growth and reproduction, i.e. division of cells.
The term "cell division" means mitosis, i.e. the usual process of cell reproduction.

The terms "gene(s)", "polynucleotide", "nucleic acid sequence", "nucleotide sequence", "DNA sequence" or "nucleic acid molecule(s)" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA, and RNA. It also includes known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occuring nucleotides with an analog. Preferably, the DNA sequence of the invention comprises a coding sequence encoding the above defined mitogenic cyclin.

A "coding sequence" is a nucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to mRNA, cDNA, recombinant nucleotide sequences or genomic DNA, while introns may be present as well under certain circumstances.

In accordance with the present invention a two-hybrid system (Fields et al., Nature 340 (1989), 245-246,) was exploited whereby CDC2aAt as bait and a cDNA library of a cell suspension as prey are used. The library was made from a mixture mRNA from Arabidopsis thaliana cell suspensions harvested at various growing stages: early exponential, exponential, early stationary and stationary phase. A positive clone was identified. The clone was first designated as LDV 59 and encodes a novel mitogenic cyclin. According to the nomenclature in Renaudin et al., Plant Mol. Biol. 32 (1996), 1003-1018, the novel mitogenic gene of the invention is also referred to herein as *CYCD4;1*, a novel class of D-type cyclins.

The *CYCD4;1* gene contains a 5' untranslated region of 117 bp. Because CYCD4;1 was isolated as a fusion protein with the GAL4 activation domain, no upstream in-

frame stop codons are present. However, alignment of CYCD4;1 with the other *A. thaliana* D-type cyclins suggests that the CYCD4;1 sequence is full length (SEQ ID NO: 1). Moreover, the initiation codon at position 118 contains a good consensus translation start sequence (GxxAUGG; Kozak 1987, J. Mol. Biol. 196, p947-950). In the 3' untranslated region of 121 bp, two overlapping polyadenylation signals can be recognized. The *CYCD4;1*-encoded protein of 308 amino acids (SEQ ID NO: 2) has a calculated molecular mass of approximately 34 kDa and bears an Rb-interaction motif in its amino terminus (amino acids 10 to 14). Using the PESTFIND program (Rogers *et al.* 1986, Science 234, p364-368), a PEST sequence was located between amino acids 1 to 93 (PESTFIND score +2.9). PEST sequences (rich in proline, glutamic acid, serine, and threonine) are characteristically present in unstable proteins.

The CYCD4;1 protein only shows significant sequence similarity to the other *A. thaliana* D-type cyclins within its amino-terminal domain, especially the cyclin box (Figure 1). In this region, CYCD4;1 is 61.3%; 69.8%; and 66.6% identical with CYCD1, CYCD2, and CYCD3, respectively. Considering the complete protein, CYCD4;1 exhibits 37.2%, 44.4%, and 31.9% identity with CYCD1;1, CYCD2;1, and CYCD3;1, respectively (Table 1).

Table 1. Sequence similarity and identity (bold) between the different *A. thaliana* D-type cyclins.

	CYCD1	CYCD2	CYCD3	CYCD4
CYCD1	-	41.6	43.4	45.3
CYCD2	**36.5**	-	38.5	52.3
CYCD3	**32.5**	**26.7**	-	46.6
CYCD4	**37.2**	**44.4**	**31.9**	-

The CYCD4;1 protein is most closely related to CYCD2;1, although their sequence similarity is limited to 52.3% (Table 1). The carboxyl-terminal domain of CYCD4;1 is also significantly shorter than that of CYCD2;1, resulting in a remarkable difference in protein size (34 kDa and 43 kDa for CYCD4;1 and CYCD2;1, respectively), and consequently in the putative protein structure. Therefore, CYCD2;1 and CYCD4;1 can be considered as members of different groups.

Thus it is evident that the gene comprising the nucleotide sequence of SEQ ID NO.1 encodes a member of a novel class of mitogenic cyclins.

The present invention also relates to DNA sequences hybridizing with the above-described DNA sequences and differ in one or more positions in comparison with these as long as they encode a mitogenic cyclin. By "hybridizing" it is meant that such nucleic acid molecules hybridize under conventional hybridization conditions, preferably under stringent conditions such as described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)). An example of one such stringent hybridization condition is hybridization at 4XSSC at 65 °C, followed by a washing in 0.1XSSC at 65 °C for one hour. Alternatively, an exemplary stringent hybridization condition is in 50 % formamide, 4XSSC at 42 °C. Mitogenic cyclins derived from other organisms such as mammals, in particular humans, may be encoded by other DNA sequences which hybridize to the sequences for plant mitogenic cyclins under relaxed hybridization conditions and which code on expression for peptides having the ability to interact with cell cycle proteins. Examples of such non-stringent hybridization conditions are 4XSSC at 50 °C or hybridization with 30-40 % formamide at 42 °C. Such molecules comprise those which are fragments, analogues or derivatives of the mitogenic cyclin of the invention and differ, for example, by way of amino acid and/or nucleotide deletion(s), insertion(s), substitution(s), addition(s) and/or recombination(s) or any other modification(s) known in the art either alone or in combination from the above-described amino acid sequences or their underlying nucleotide sequence(s). Methods for introducing such modifications in the nucleic acid molecules according to the invention are well-known to the person skilled in the art. The invention also relates to nucleic acid molecules the sequence of which differs from the nucleotide sequence of any of the above-described nucleic acid molecules due to the degeneracy of the genetic code. All such fragments, analogues and derivatives of the protein of the invention are included within the scope of the present invention, as long as the essential characteristic immunological and/or biological properties as

defined above remain unaffected in kind, that is the novel nucleic acid molecules of the invention include all nucleotide sequences encoding proteins or peptides which have at least a part of the primary structural conformation for one or more epitopes capable of reacting with antibodies to mitogenic cyclins which are encodable by a nucleic acid molecule as set forth above and which have comparable or identical characteristics in terms of biological activity and/or the capability to interact with other proteins. Part of the invention is therefore also nucleic acid molecules encoding a polypeptide comprising at least a functional part of a mitogenic cyclin encoded by a nucleic acid sequence comprised in a nucleic acid molecule according to the invention. An example for this is that the polypeptide or a fragment thereof according to the invention is embedded in another amino acid sequence. Preferably, the DNA sequence of the invention encodes a protein having substantially the same amino acid sequence as the protein defined in SEQ ID NO 2.

As is demonstrated in the appended examples a two-hybrid screening assay has been developed in accordance with the present invention suitable for identifying mitogenic cyclins. Thus, in another aspect the present invention relates to a method for identifying and obtaining mitogenic cyclins comprising a two-hybrid screening assay wherein CDC2a as a bait and a cDNA library of cell suspension culture as prey are used. Preferably, said CDC2a is CDC2aAt. However, PEST containing CDKs from other plants and/or other organisms such as mammals may be employed as well. The cell culture may be from any organism possessing mitogenic cyclins such as animals, preferably mammals. Particularly preferred are plant cell suspension cultures such as from Arabidopsis.

The nucleic acid molecules encoding proteins or peptides identified to interact with the CDC2a in the above mentioned assay can be easily obtained and sequenced by methods known in the art; see also the appended examples. Therefore, the present invention also relates to a DNA sequence encoding a mitogenic cyclin obtainable by the method of the invention.

In a preferred embodiment the nucleic acid molecules according to the invention are RNA or DNA molecules, preferably cDNA, genomic DNA or synthetically synthesized DNA or RNA molecules. Preferably, the nucleic acid molecule of the invention is derived from a plant, preferably from *Arabidopsis thaliana*. However, since mitogenic cyclins are supposed to play a key role in plant cell division, corresponding proteins displaying similar properties should be present in other plants as well. Nucleic acid molecules of the invention can be obtained, e.g., by hybridization of the above-described nucleic acid molecules with a (sample of) nucleic acid molecule(s) of any source. Nucleic acid molecules hybridizing with the above-described nucleic acid molecules can in general be derived from any organism, preferably plants possessing such molecules, preferably from monocotyledonous or dicotyledonous plants, in particular from plants of interest in agriculture, horticulture or wood culture, such as crop plants, namely those of the family Poaceae, any starch producing plants, such as potato, maniok, leguminous plants, oil producing plants, such as oilseed rape, linenseed, etc., plants using polypeptide as storage substances, such as soybean, plants using sucrose as storage substance, such as sugar beet or sugar cane, trees, ornamental plants etc. Preferably, the nucleic acid molecules according to the invention are derived from *Arabidopsis thaliana*. Nucleic acid molecules hybridizing to the above-described nucleic acid molecules can be isolated, e.g., form libraries, such as cDNA or genomic libraries by techniques well known in the art. For example, hybridizing nucleic acid molecules can be identified and isolated by using the above-described nucleic acid molecules or fragments thereof or complements thereof as probes to screen libraries by hybridizing with said molecules according to standard techniques. Possible is also the isolation of such nucleic acid molecules by applying a nucleic acid amplicification technique such as the polymerase chain reaction (PCR) using as primers oligonucleotides derived form the above-described nucleic acid molecules.

Nucleic acid molecules which hybridize with any of the aforementioned nucleic acid molecules also include fragments, derivatives and allelic variants of the above-described nucleic acid molecules that encode a mitogenic cyclin or an immunologically or functional fragment thereof. Fragments are understood to be

parts of nucleic acid molecules long enough to encode the described protein or a functional or immunologically active fragment thereof as defined above. Preferably, the functional fragment contains at least the cyclin box of the CYCD4 protein shown in Figure 1. Preferably, the fragment comprises amino acid residues 78 to 182 of the amino acid sequence of SEQ ID NO:2.

The term "derivative" means in this context that the nucleotide sequence of these nucleic acid molecules differs from the sequences of the above-described nucleic acid molecules in one or more nucleotide positions and are highly homologous to said nucleic acid molecules. Homology is understood to refer to a sequence identity of at least 40 %, particularly an identity of at least 60 %, preferably more than 80 % and still more preferably more than 90 %. The term "substantially homologous" refers to a subject, for instance a nucleic acid, which is at least 50% identical in sequence to the reference when the entire ORF (open reading frame) is compared, where the sequence identity is preferably at least 70%, more preferably at least 80%, still more preferably at least 85%, especially more than about 90%, most preferably 95% or greater, particularly 98% or greater. The deviations from the sequences of the nucleic acid molecules described above can, for example, be the result of nucleotide substitution(s), deletion(s), addition(s), insertion(s) and/or recombination(s); see supra.

Homology further means that the respective nucleic acid molecules or encoded proteins are functionally and/or structurally equivalent. The nucleic acid molecules that are homologous to the nucleic acid molecules described above and that are derivatives of said nucleic acid molecules are, for example, variations of said nucleic acid molecules which represent modifications having the same biological function, in particular encoding proteins with the same or substantially the same biological function. They may be naturally occurring variations, such as sequences from other plant varieties or species, or mutations. These mutations may occur naturally or may be obtained by mutagenesis techniques. The allelic variations may be naturally occurring allelic variants as well as synthetically produced or genetically engineered variants; see supra.

The proteins encoded by the various derivatives and variants of the above-described nucleic acid molecules may share specific common characteristics, such as biological activity, molecular weight, immunological reactivity, conformation, etc., as well as physical properties, such as electrophoretic mobility, chromatographic behavior, sedimentation coefficients, pH optimum, temperature optimum, stability, solubility, spectroscopic properties, etc.

Examples of the different possible applications of the nucleic acid molecules according to the invention as well as molecules derived from them will be described in detail in the following.

Hence, in a further embodiment, the invention relates to nucleic acid molecules of at least 15 nucleotides in length hybridizing specifically with a nucleic acid molecule as described above or with a complementary strand thereof. Specific hybridization occurs preferably under stringent conditions and implies no or very little cross-hybridization with nucleotide sequences encoding no or substantially different proteins. Such nucleic acid molecules may be used as probes and/or for the control of gene expression. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary in length. Preferred are nucleic acid probes of 16 to 35 nucleotides in length. Of course, it may also be appropriate to use nucleic acids of up to 100 and more nucleotides in length. The nucleic acid probes of the invention are useful for various applications. On the one hand, they may be used as PCR primers for amplification of nucleic acid sequences according to the invention. The design and use of said primers is known by the person skilled in the art. Preferably such amplification primers comprise a contiguous sequence of at least 6 nucleotides, in particular 13 nucleotides, preferably 15 to 25 nucleotides or more, identical or complementary to the nucleotide sequence depicted in SEQ ID NO: 1 or to a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 2. Another application is the use as a hybridization probe to identify nucleic acid molecules hybridizing with a nucleic acid molecule of the invention by homology screening of genomic DNA or cDNA libraries. Nucleic acid molecules according to this preferred embodiment of the

invention which are complementary to a nucleic acid molecule as described above may also be used for repression of expression of a cell cycle gene, for example due to an antisense or triple helix effect or for the construction of appropriate ribozymes (see, e.g., EP-A1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a gene comprising a nucleic acid molecule of the invention or part thereof. Selection of appropriate target sites and corresponding ribozymes can be done as described, for example, in Steinecke, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds Academic Press, Inc. (1995), 449-460. Furthermore, the person skilled in the art is well aware that it is also possible to label such a nucleic acid probe with an appropriate marker for specific applications, such as for the detection of the presence of a nucleic acid molecule of the invention in a sample derived from an organism, in particular plants.

The above described nucleic acid molecules may either be DNA or RNA or a hybrid thereof. Furthermore, said nucleic acid molecule may contain, for example, thioester bonds and/or nucleotide analogues, commonly used in oligonucleotide anti-sense approaches. Said modifications may be useful for the stabilization of the nucleic acid molecule against endo- and/or exonucleases in the cell. Said nucleic acid molecules may be transcribed by an appropriate vector containing a chimeric gene which allows for the transcription of said nucleic acid molecule in the cell.

Furthermore, the so-called "peptide nucleic acid" (PNA) technique can be used for the detection or inhibition of the expression of a nucleic acid molecule of the invention. For example, the binding of PNAs to complementary as well as various single stranded RNA and DNA nucleic acid molecules can be systematically investigated using thermal denaturation and BIAcore surface-interaction techniques (Jensen, Biochemistry 36 (1997), 5072-5077). Furthermore, the nucleic acid molecules described above as well as PNAs derived therefrom can be used for detecting point mutations by hybridization with nucleic acids obtained from a sample with an affinity sensor, such as BIAcore; see Gotoh, Rinsho Byori 45 (1997), 224-228. Hybridization based DNA screening on peptide nucleic acids (PNA) oligomer arrays are described in the prior art, for example in Weiler, Nucleic Acids Research

25 (1997), 2792-2799. The synthesis of PNAs can be performed according to methods known in the art, for example, as described in Koch, J. Pept. Res. 49 (1997), 80-88; Finn, Nucleic Acids Research 24 (1996), 3357-3363. Further possible applications of such PNAs, for example as restriction enzymes or as templates for the synthesis of nucleic acid oligonucleotides are known to the person skilled in the art and are, for example, described in Veselkov, Nature 379 (1996), 214 and Bohler, Nature 376 (1995), 578-581.

The present invention also relates to vectors, particularly plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering that contain a nucleic acid molecule according to the invention. Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the nucleic acid molecules and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

In a preferred embodiment the nucleic acid molecule present in the vector is linked to (a) control sequence(s) which allow the expression of the nucleic acid molecule in prokaryotic and/or eukaryotic cells.
The term "control sequence" refers to regulatory DNA sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, control sequences generally include promoter, ribosomal binding site, and terminators. In eukaryotes generally control sequences include promoters, terminators and, in some instances, enhancers, transactivators or transcription factors. The term "control sequence" is intended to include, at a minimum, all components the presence of which are necessary for expression, and may also include additional advantageous components.

The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. In case the control sequence is a promoter, it is obvious for a skilled person that double-stranded nucleic acid is used.

Thus, the vector of the invention is preferably an expression vector. An "expression vector" is a construct that can be used to transform a selected host cell and provides for expression of a coding sequence in the selected host. Expression vectors can for instance be cloning vectors, binary vectors or integrating vectors. Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in prokaryotic and/or eukaryotic cells are well known to those skilled in the art. In the case of eukaryotic cells they comprise normally promoters ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript, for example, those of the 35S RNA from Cauliflower Mosaic Virus (CaMV). Other promoters commonly used are the polyubiquitin promoter, and the actin promoter for ubiquitous expression. The termination signals usually employed are from the Nopaline Synthase promoter or from the CAMV 35S promoter. A plant translational enhancer often used is the TMV omega sequences, the inclusion of an intron (Intron-1 from the Shrunken gene of maize, for example) has been shown to increase expression levels by up to 100-fold. (Mait, Transgenic Research 6 (1997), 143-156; Ni, Plant Journal 7 (1995), 661-676). Additional regulatory elements may include transcriptional as well as translational enhancers. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the P_L, *lac*, *trp* or *tac* promoter in *E. coli*, and examples of regulatory elements permitting expression in eukaryotic host cells are the *AOX1* or *GAL1* promoter in yeast or the CMV-, SV40-, RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), pSPORT1 (GIBCO BRL). Advantageously, the above-described vectors of the

invention comprises a selectable and/or scorable marker. Selectable marker genes useful for the selection of transformed plant cells, callus, plant tissue and plants are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for dhfr, which confers resistance to methotrexate (Reiss, Plant Physiol. (Life Sci. Adv.) 13 (1994), 143-149); npt, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, EMBO J. 2 (1983), 987-995) and hygro, which confers resistance to hygromycin (Marsh, Gene 32 (1984), 481-485). Additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); mannose-6-phosphate isomerase which allows cells to utilize mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) or deaminase from Aspergillus terreus which confers resistance to Blasticidin S (Tamura, Biosci. Biotechnol. Biochem. 59 (1995), 2336-2338).
Useful scorable marker are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, Pl. Sci. 116 (1996), 59-72; Scikantha, J. Bact. 178 (1996), 121), green fluorescent protein (Gerdes, FEBS Lett. 389 (1996), 44-47) or ß-glucuronidase (Jefferson, EMBO J. 6 (1987), 3901-3907). This embodiment is particularly useful for simple and rapid screening of cells, tissues and organisms containing a vector of the invention.

The present invention furthermore relates to host cells comprising a vector as described above or a nucleic acid molecule according to the invention wherein the nucleic acid molecule is foreign to the host cell.
By "foreign" it is meant that the nucleic acid molecule is either heterologous with respect to the host cell, this means derived from a cell or organism with a different genomic background, or is homologous with respect to the host cell but located in a different genomic environment than the naturally occurring counterpart of said

nucleic acid molecule. This means that, if the nucleic acid molecule is homologous with respect to the host cell, it is not located in its natural location in the genome of said host cell, in particular it is surrounded by different genes. In this case the nucleic acid molecule may be either under the control of its own promoter or under the control of a heterologous promoter. The vector or nucleic acid molecule according to the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained in some form extrachromosomally. In this respect, it is also to be understood that the nucleic acid molecule of the invention can be used to restore or create a mutant gene via homologous recombination (Paszkowski (ed.), Homologous Recombination and Gene Silencing in Plants. Kluwer Academic Publishers (1994)).

The host cell can be any prokaryotic or eukaryotic cell, such as bacterial, insect, fungal, plant or animal cells. Preferred fungal cells are, for example, those of the genus Saccharomyces, in particular those of the species S. cerevisiae.

Another subject of the invention is a method for the preparation of mitogenic cyclins which comprises the cultivation of host cells according to the invention which, due to the presence of a vector or a nucleic acid molecule according to the invention, are able to express such a protein, under conditions which allow expression of the protein and recovering of the so-produced protein from the culture.
The term "expression" means the production of a protein or nucleotide sequence in the cell. However, said term also includes expression of the protein in a cell-free system. It includes transcription into an RNA product, post-transcriptional modification and/or translation to a protein product or polypeptide from a DNA encoding that product, as well as possible post-translational modifications. Depending on the specific constructs and conditions used, the protein may be recovered from the cells, from the culture medium or from both. For the person skilled in the art it is well known that it is not only possible to express a native protein but also to express the protein as fusion polypeptides or to add signal sequences directing the protein to specific compartments of the host cell, e.g., ensuring secretion of the protein into the culture medium, etc. Furthermore, such a protein

and fragments thereof can be chemically synthesized and/or modified according to standard methods described, for example hereinbelow.

The terms "protein" and "polypeptide" used in this application are interchangeable. "Polypeptide" refers to a polymer of amino acids (amino acid sequence) and does not refer to a specific length of the molecule. Thus peptides and oligopeptides are included within the definition of polypeptide. This term does also refer to or include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The present invention furthermore relates to proteins encoded by the nucleic acid molecules according to the invention or produced or obtained by the above-described methods, and to functional and/or immunologically active fragments of such mitogenic cyclins. The proteins and polypeptides of the present invention are not necessarily translated from a designated nucleic acid sequence; the polypeptides may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system, or isolation from a suitable viral system. The polypeptides may include one or more analogs of amino acids, phosphorylated amino acids or unnatural amino acids. Methods of inserting analogs of amino acids into a sequence are known in the art. The polypeptides may also include one or more labels, which are known to those skilled in the art. In this context, it is also understood that the proteins according to the invention may be further modified by conventional methods known in the art. By providing the proteins according to the present invention it is also possible to determine fragments which retain biological activity. This allows the construction of chimeric proteins and peptides comprising an amino sequence derived from the protein of the invention, which is crucial for its, e.g., binding activity and other functional amino acid sequences, e.g. GUS marker gene (Jefferson, EMBO J. 6 (1987), 3901-3907). The other functional amino acid sequences may be either physically linked by, e.g.,

chemical means to the proteins of the invention or may be fused by recombinant DNA techniques well known in the art.

The term "fragment of a sequence" or "part of a sequence" means a truncated sequence of the original sequence referred to. The truncated sequence (nucleic acid or protein sequence) can vary widely in length; the minimum size being a sequence of sufficient size to provide a sequence with at least a comparable function and/or activity of the original sequence referred to, while the maximum size is not critical. In some applications, the maximum size usually is not substantially greater than that required to provide the desired activity and/or function(s) of the original sequence. Typically, the truncated amino acid sequence will range from about 5 to about 60 amino acids in length. More typically, however, the sequence will be a maximum of about 50 amino acids in length, preferably a maximum of about 30 amino acids. It is usually desirable to select sequences of at least about 10, 12 or 15 amino acids, up to a maximum of about 20 or 25 amino acids.

Furthermore, folding simulations and computer redesign of structural motifs of the protein of the invention can be performed using appropriate computer programs (Olszewski, Proteins 25 (1996), 286-299; Hoffman, Comput. Appl. Biosci. 11 (1995), 675-679). Computer modeling of protein folding can be used for the conformational and energetic analysis of detailed peptide and protein models (Monge, J. Mol. Biol. 247 (1995), 995-1012; Renouf, Adv. Exp. Med. Biol. 376 (1995), 37-45). In particular, the appropriate programs can be used for the identification of interactive sites of mitogenic cyclin and its receptor, its ligand or other interacting proteins by computer assistant searches for complementary peptide sequences (Fassina, Immunomethods 5 (1994), 114-120. Further appropriate computer systems for the design of protein and peptides are described in the prior art, for example in Berry, Biochem. Soc. Trans. 22 (1994), 1033-1036; Wodak, Ann. N. Y. Acad. Sci. 501 (1987), 1-13; Pabo, Biochemistry 25 (1986), 5987-5991. The results obtained from the above-described computer analysis can be used for, e.g., the preparation of peptidomimetics of the protein of the invention or fragments thereof. Such pseudopeptide analogues of the natural amino acid sequence of the protein may very efficiently mimic the parent protein (Benkirane, J. Biol. Chem. 271 (1996), 33218-33224). For example, incorporation of easily available achiral Ω-amino acid residues into a protein of the

invention or a fragment thereof results in the substitution of amide bonds by polymethylene units of an aliphatic chain, thereby providing a convenient strategy for constructing a peptidomimetic (Banerjee, Biopolymers 39 (1996), 769-777). Superactive peptidomimetic analogues of small peptide hormones in other systems are described in the prior art (Zhang, Biochem. Biophys. Res. Commun. 224 (1996), 327-331). Appropriate peptidomimetics of the protein of the present invention can also be identified by the synthesis of peptidomimetic combinatorial libraries through successive amide alkylation and testing the resulting compounds, e.g., for their binding and immunological properties. Methods for the generation and use of peptidomimetic combinatorial libraries are described in the prior art, for example in Ostresh, Methods in Enzymology 267 (1996), 220-234 and Dorner, Bioorg. Med. Chem. 4 (1996), 709-715.

Furthermore, a three-dimensional and/or crystallographic structure of the protein of the invention can be used for the design of peptidomimetic inhibitors of the biological activity of the protein of the invention (Rose, Biochemistry 35 (1996), 12933-12944; Rutenber, Bioorg. Med. Chem. 4 (1996), 1545-1558).

Furthermore, the present invention relates to antibodies specifically recognizing a mitogenic cyclin according to the invention or parts, i.e. specific fragments or epitopes, of such a protein. The antibodies of the invention can be used to identify and isolate other mitogenic cyclins and genes in any organism, preferably plants. These antibodies can be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv or scFv fragments etc. Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, Nature 256 (1975), 495, and Galfré, Meth. Enzymol. 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals. Furthermore, antibodies or fragments thereof to the aforementioned peptides can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. These antibodies can be used, for example, for the immunoprecipitation and immunolocalization of proteins according to the invention as well as for the monitoring of the synthesis of such proteins, for example, in

recombinant organisms, and for the identification of compounds interacting with the protein according to the invention. For example, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies selections, yielding a high increment of affinity from a single library of phage antibodies which bind to an epitope of the protein of the invention (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmborg, J. Immunol. Methods 183 (1995), 7-13). In many cases, the binding phenomena of antibodies to antigens is equivalent to other ligand/anti-ligand binding.

Plant cell division can conceptually be influenced in three ways : (i) inhibiting or arresting cell division, (ii) maintaining, facilitating or stimulating cell division or (iii) uncoupling DNA synthesis from mitosis and cytokinesis. Modulation of the expression of a mitogenic cyclin encoded by a nucleotide sequence according to the invention has surprisingly an advantageous influence on plant cell division characteristics, in particular on the disruption of the expression levels of genes involved in G1/S and/or G2/M transition and as a result therof on the total make-up of the plant concerned or parts thereof. An example is that DNA synthesis or progression of DNA replication will be negatively influenced by interfering with the formation of a cyclin-dependent protein kinase complex. Alternatively, overexpression of the mitogen cyclin accelerates reentry into the cell cycle.
The term "cyclin-dependent protein kinase complex" means the complex formed when a, preferably functional, cyclin associates with a, preferably, functional cyclin dependent kinase. Such complexes may be active in phosphorylating proteins and may or may not contain additional protein species.
The term "protein kinase" means an enzyme catalyzing the phosphorylation of proteins.

To analyse the industrial applicabilities of the invention, transformed plants can be made overproducing the nucleotide sequence according to the invention. Such an overexpression of the new gene(s), proteins or inactivated variants thereof will either positively or negatively have an effect on cell division. Methods to modify the expression levels and/or the activity are known to persons skilled in the art and

include for instance overexpression, co-suppression, the use of ribozymes, sense and anti-sense strategies, gene silencing approaches. "Sense strand" refers to the strand of a double-stranded DNA molecule that is homologous to a mRNA transcript thereof. The "anti-sense strand" contains an inverted sequence which is complementary to that of the "sense strand".

Hence, the nucleic acid molecules according to the invention are in particular useful for the genetic manipulation of plant cells in order to modify the characteristics of plants and to obtain plants with modified, preferably with improved or useful phenotypes. Similarly, the invention can also be used to modulate the cell division and the growth of cells, preferentially plant cells, in *in vitro* cultures. Specifically the plant cell division rate and/or the inhibition of a plant cell division can be influenced by overexpression or reducing the expression of a gene encoding a protein according to the invention. Overexpression of a cyclin gene according to the invention promotes cell proliferation, while reducing cyclin expression arrests cell division or prevents reentry into the cell cycle. Part of the invention is thus the usage of a cyclin comprising the coding sequence or part thereof as mentioned hereinbefore as a negative or positive regulator of cell proliferation.

As a result of overproduction the G1/S generation time is shortened whereas the proliferation is less dependent on growth factors. A transformed plant can thus be obtained by transforming a plant cell with a gene encoding a polypeptide concerned or fragment thereof alone or in combination, whereas the plant cell may belong to a monocotyledonous or dicotyledonous plant. For this purpose tissue specific promoters, in one construct or being present as a separate construct in addition to the sequence concerned, can be used. Alternatively the expression of the cyclin is inducible by cytokinines or sucrose.

Surprisingly using a polypeptide or fragment thereof according to the invention or using antisense RNA for the gene according to the invention cell division of the meristems of the plant can be manipulated, positively and/or negatively respectively. Furthermore, overproduction of the cyclin enhances growth and results in cell division to be less sensitive to an arrest caused by environmental stress such as salt, drought, chilling and the like.

Thus, the present invention provides a method for the production of transgenic plants, plant cells or plant tissue comprising the introduction of a nucleic acid molecule or vector of the invention into the genome of said plant, plant cell or plant tissue.

For the expression of the nucleic acid molecules according to the invention in sense or antisense orientation in plant cells, the molecules are placed under the control of regulatory elements which ensure the expression in plant cells. These regulatory elements may be heterologous or homologous with respect to the nucleic acid molecule to be expressed as well with respect to the plant species to be transformed. In general, such regulatory elements comprise a promoter active in plant cells. To obtain expression in all tissues of a transgenic plant, preferably constitutive promoters are used, such as the 35 S promoter of CaMV (Odell, Nature 313 (1985), 810-812) or promoters of the polyubiquitin genes of maize (Christensen, Plant Mol. Biol. 18 (1982), 675-689). In order to achieve expression in specific tissues of a transgenic plant it is possible to use tissue specific promoters (see, e.g., Stockhaus, EMBO J. 8 (1989), 2245-2251). Known are also promoters which are specifically active in tubers of potatoes or in seeds of different plants species, such as maize, Vicia, wheat, barley etc. Inducible promoters may be used in order to be able to exactly control expression. An example for inducible promoters are the promoters of genes encoding heat shock proteins. Also microspore-specific regulatory elements and their uses have been described (WO96/16182). Furthermore, the chemically inducible Tet-system may be employed (Gatz, Mol. Gen. Genet. 227 (1991); 229-237). Further suitable promoters are known to the person skilled in the art and are described, e.g., in Ward (Plant Mol. Biol. 22 (1993), 361-366). The regulatory elements may further comprise transcriptional and/or translational enhancers functional in plants cells. Furthermore, the regulatory elements may include transcription termination signals, such as a poly-A signal, which lead to the addition of a poly A tail to the transcript which may improve its stability.

In the case that a nucleic acid molecule according to the invention is expressed in sense orientation it is in principle possible to modify the coding sequence in such a way that the protein is located in any desired compartment of the plant cell. These include the nucleus, endoplasmatic reticulum, the vacuole, the mitochondria, the plastids, the apoplast, the cytoplasm etc. Since the interacting component of the protein of the invention excerts its effects in the cytoplasm and/or nucleus, corresponding signal sequences are preferred to direct the protein of the invention in the same compartment. Methods how to carry out this modifications and signal sequences ensuring localization in a desired compartment are well known to the person skilled in the art.

Methods for the introduction of foreign DNA into plants are also well known in the art. These include, for example, the transformation of plant cells or tissues with T-DNA using Agrobacterium tumefaciens or Agrobacterium rhizogenes, the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, biolistic methods like particle bombardment, pollen-mediated transformation, plant RNA virus-mediated transformation, liposome-mediated transformation, transformation using wounded or enzyme-degraded immature embryos, or wounded or enzyme-degraded embryogenic callus and other methods known in the art. The vectors used in the method of the invention may contain further functional elements, for example "left border"- and "right border"-sequences of the T-DNA of Agrobacterium which allow for stably integration into the plant genome. Furthermore, methods and vectors are known to the person skilled in the art which permit the generation of marker free transgenic plants, i.e. the selectable or scorable marker gene is lost at a certain stage of plant development or plant breeding. This can be achieved by, for example cotransformation (Lyznik, Plant Mol. Biol. 13 (1989), 151-161; Peng, Plant Mol. Biol. 27 (1995), 91-104) and/or by using systems which utilize enzymes capable of promoting homologous recombination in plants (see, e.g., WO97/08331; Bayley, Plant Mol. Biol. 18 (1992), 353-361); Lloyd, Mol. Gen. Genet. 242 (1994), 653-657; Maeser, Mol. Gen. Genet. 230 (1991), 170-176; Onouchi, Nucl. Acids Res. 19 (1991), 6373-6378). Methods for the preparation of appropriate

vectors are described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Suitable strains of Agrobacterium tumefaciens and vectors as well as transformation of Agrobacteria and appropriate growth and selection media are well known to those skilled in the art and are described in the prior art (GV3101 (pMK90RK), Koncz, Mol. Gen. Genet. 204 (1986), 383-396; C58C1 (pGV 3850kan), Deblaere, Nucl. Acid Res. 13 (1985), 4777; Bevan, Nucleic. Acid Res. 12(1984), 8711; Koncz, Proc. Natl. Acad. Sci. USA 86 (1989), 8467-8471; Koncz, Plant Mol. Biol. 20 (1992), 963-976; Koncz, Specialized vectors for gene tagging and expression studies. In: Plant Molecular Biology Manual Vol 2, Gelvin and Schilperoort (Eds.), Dordrecht, The Netherlands: Kluwer Academic Publ. (1994), 1-22; EP-A-120 516; Hoekema: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V, Fraley, Crit. Rev. Plant. Sci., 4, 1-46; An, EMBO J. 4 (1985), 277-287). Although the use of Agrobacterium tumefaciens is preferred in the method of the invention, other Agrobacterium strains, such as Agrobacterium rhizogenes, may be used, for example if a phenotype conferred by said strain is desired.

Methods for the transformation using biolistic methods are well known to the person skilled in the art; see, e.g., Wan, Plant Physiol. 104 (1994), 37-48; Vasil, Bio/Technology 11 (1993), 1553-1558 and Christou (1996) Trends in Plant Science 1, 423-431. Microinjection can be performed as described in Potrykus and Spangenberg (eds.), Gene Transfer To Plants. Springer Verlag, Berlin, NY (1995).

The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods as, e.g., described above as well as protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, etc.

The term "transformation" as used herein, refers to the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for the transfer. The polynucleotide may be transiently or stably introduced into the host cell and may be maintained non-integrated, for example, as a plasmid or as chimeric links, or alternatively, may be integrated into the host genome. The resulting transformed

plant cell can then be used to regenerate a transformed plant in a manner known by a skilled person.

In general, the plants which can be modified according to the invention and which either show overexpression of a protein according to the invention or a reduction of the synthesis of such a protein can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture interest, such as crop plants (e.g. maize, rice, barley, wheat, rye, oats etc.), potatoes, oil producing plants (e.g. oilseed rape, sunflower, pea nut, soy bean, etc.), cotton, sugar beet, sugar cane, leguminous plants (e.g. beans, peas etc.), wood producing plants, preferably trees, etc.

Thus, the present invention relates also to transgenic plant cells which contain (preferably stably integrated into the genome) a nucleic acid molecule according to the invention linked to regulatory elements which allow expression of the nucleic acid molecule in plant cells and wherein the nucleic acid molecule is foreign to the transgenic plant cell. For the meaning of foreign; see supra.

The presence and expression of the nucleic acid molecule in the transgenic plant cells leads to the synthesis of a mitogenic cyclin and leads to physiological and phenotypic changes in plants containing such cells.

Thus, the present invention also relates to transgenic plants and plant tissue comprising transgenic plant cells according to the invention. Due to the (over)expression of a mitogenic cyclin of the invention, e.g., at developmental stages and/or in plant tissue in which they do not naturally occur these transgenic plants may show various physiological, developmental and/or morphological modifications in comparison to wild-type plants.
For example, to obtain transgenic plants overexpressing the A. thaliana CYCD4;1 gene, its coding region can be cloned, e.g., into the pAT7002 vector (Aoyama and Chua, 997, Plant J. 11, p605-612). This vector allows inducible expression of the

cloned inserts by the addition of the glucocorticoid dexamethasone. For example, following a polymerase chain reaction (PCR) technology the coding region of CYCD4;1 can be amplified using 5'-GAACACTCGAGTGTAATGGCAGAGG-3' (SEQ ID NO: 3) and 5'-CATCATACTAGTTATAATAATGTAAG-3' (SEQ ID NO: 4) primers. The obtained PCR fragment can be purified and cut with XhoI and SpeI. Subsequently the fragment can be cloned into the XhoI and SpeI sites of pTA7002. The resulted binary vector can be transferred into Agrobacterium tumefaciens. This strain can be used to transform Nicotiana tabacum cv. Petit havana using, e.g., the leaf disk protocol (Horsh et al., 1985, Science 227, p1229-1231) and Arabidopsis thaliana using, e.g., the root transformation protocol (Valvekens et al., 1988, PNAS 85, 5536-5540). Transgenic plants can then be selected on hygromycine 20 mg/l.

Plants can be tested for CYCD4 inducible expression as follows. 2 to 3 leaves of each transformant can be cut in two. Each half can be either submersed in 50 mM Na-citrate buffer (pH 5.8) with or without dexamethasone (0.03 mM concentration). After 24 hours of induction RNA can be extracted from these leaves using the Trizol reagents (Gibco-BRL) according to the manufactures and a northern gel can be run using, e.g., 5 μg of RNA. The gel can be blotted on a nitro-cellulose filter (HybondN+, Amersham) and hybridised with a CYCD4 probe.

Furthermore, seeds of transformants can be put on ½ MS medium with 1% sucrose, both with and without dexamethasone. As a control SR1 seeds should be included. In the presence of dexamethasone the growth behaviour of the transgenic plants as compared to the control plants is expected to be modified. For example, these transgenic plants may grow faster and/or have additional cells.
Furthermore, said plant may be less sensitive to environmental stress compared to the corresponding wild type plant.

Furthermore, the invention also relates to a transgenic plant cell which contains (preferably stably integrated into the genome) a nucleic acid molecule according to the invention or part thereof, wherein the transcription and/or expression of the nucleic

acid molecule or part thereof leads to reduction of the synthesis of a cell cycle interacting protein.

In a preferred embodiment, the reduction is achieved by an anti-sense, sense, ribozyme, co-suppression and/or dominant mutant effect.
"Antisense" and "antisense nucleotides" means DNA or RNA constructs which block the expression of the naturally occurring gene product.
The provision of the nucleic acid molecules according to the invention opens up the possibility to produce transgenic plant cells with a reduced level of the protein as described above and, thus, with a defect in cell division. Techniques how to achieve this are well known to the person skilled in the art. These include, for example, the expression of antisense-RNA, ribozymes, of molecules which combine antisense and ribozyme functions and/or of molecules which provide for a co-suppression effect; see also supra. When using the antisense approach for reduction of the amount of mitogenic cyclins in plant cells, the nucleic acid molecule encoding the antisense-RNA is preferably of homologous origin with respect to the plant species used for transformation. However, it is also possible to use nucleic acid molecules which display a high degree of homology to endogenously occurring nucleic acid molecules encoding a mitogenic cyclin. In this case the homology is preferably higher than 80%, particularly higher than 90% and still more preferably higher than 95%.
The reduction of the synthesis of a protein according to the invention in the transgenic plant cells can result in an alteration in, e.g., cell division. In transgenic plants comprising such cells this can lead to various physiological, developmental and/or morphological changes.

Thus, the present invention also relates to transgenic plants comprising the above-described transgenic plant cells. These may show, for example, a deficiency in cell division and/or reduced growth characteristics compared to wild type plants.

The present invention also relates to cultured plant tissues comprising transgenic plant cells as described above which either show overexpression of a protein according to the invention or a reduction in synthesis of such a protein.

Any transformed plant obtained according to the invention can be used in a conventional breeding scheme or in *in vitro* plant propagation to produce more transformed plants with the same characteristics and/or can be used to introduce the same characteristic in other varieties of the same or related species. Such plants are also part of the invention. Seeds obtained from the transformed plants genetically also contain the same characteristic and are part of the invention. As mentioned before, the present invention is in principle applicable to any plant and crop that can be transformed with any of the transformation method known to those skilled in the art and includes for instance corn, wheat, barley, rice, oilseed crops, cotton, tree species, sugar beet, cassava, tomato, potato, numerous other vegetables, fruits.

In yet another aspect, the invention also relates to harvestable parts and to propagation material of the transgenic plants according to the invention which either contain transgenic plant cells expressing a nucleic acid molecule according to the invention or which contain cells which show a reduced level of the described protein. Harvestable parts can be in principle any useful parts of a plant, for example, flowers, pollen, seedlings, tubers, leaves, stems, fruit, seeds, roots etc. Propagation material includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks etc.

The present invention further relates to a method for identifying and obtaining an activator or inhibitor of mitogenic cyclins comprising the steps of:

(a) combining a compound to be screened with a reaction mixture containing the mitogenic cyclin of the invention and a readout system capable of interacting with the mitogenic cyclin under suitable conditions;

(b) maintaining said reaction mixture in the presence of the compound or a sample comprising a plurality of compounds under conditions which permit interaction of the mitogenic cyclin with said readout system;

(c) identifying or verifying a sample and compound, respectively, which leads to suppression or activation of the readout system.

The term "read out system" in context with the present invention means a DNA sequence which upon transcription and/or expression in a cell, tissue or organism provides for a scorable and/or selectable phenotype. Such read out systems are well known to those skilled in the art and comprise, for example, recombinant DNA molecules and marker genes as described above and in the appended example.
The term "plurality of compounds" in a method of the invention is to be understood as a plurality of substances which may or may not be identical.
Said compound or plurality of compounds may be chemically synthesized or microbiologically produced and/or comprised in, for example, samples, e.g., cell extracts from, e.g., plants, animals or microorganisms. Furthermore, said compound(s) may be known in the art but hitherto not known to be capable of suppressing or activating mitogenic cyclins. The reaction mixture may be a cell free extract or may comprise a cell or tissue culture. Suitable set ups for the method of the invention are known to the person skilled in the art and are, for example, generally described in Alberts et al., Molecular Biology of the Cell, third edition (1994), in particular Chapter 17. The plurality of compounds may be, e.g., added to the reaction mixture, culture medium, injected into the cell or sprayed onto the plant.
If a sample containing a compound or a plurality of compounds is identified in the method of the invention, then it is either possible to isolate the compound from the original sample identified as containing the compound capable of suppressing or activating mitogenic cyclins, or one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably said sample comprises substances of similar chemical and/or physical properties, and most preferably said substances are identical. Preferably, the compound identified according to the above described method or its derivative is further formulated in a form suitable for the application in plant breeding or plant cell and tissue culture.

The compounds which can be tested and identified according to a method of the invention may be expression libraries, e.g., cDNA expression libraries, peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, peptidomimetics, PNAs or the like (Milner, Nature Medicine 1 (1995), 879-880; Hupp, Cell 83 (1995), 237-245; Gibbs, Cell 79 (1994), 193-198 and references cited supra). Furthermore, genes encoding a putative regulator of mitogenic cyclin and/or which excert their effects up- or downstream the mitogenic cyclin of the invention may be identified using, for example, insertion mutagenesis using, for example, gene targeting vectors known in the art (see, e.g., Hayashi, Science 258 (1992), 1350-1353; Fritze and Walden, Gene activation by T-DNA tagging. In *Methods in Molecular* biology 44 (Gartland, K.M.A. and Davey, M.R., eds). Totowa: Human Press (1995), 281-294) or transposon tagging (Chandlee, Physiologia Plantarum 78 (1990), 105-115). Said compounds can also be functional derivatives or analogues of known inhibitors or activators. Methods for the preparation of chemical derivatives and analogues are well known to those skilled in the art and are described in, for example, Beilstein, Handbook of Organic Chemistry, Springer edition New York Inc., 175 Fifth Avenue, New York, N.Y. 10010 U.S.A. and Organic Synthesis, Wiley, New York, USA. Furthermore, said derivatives and analogues can be tested for their effects according to methods known in the art. Furthermore, peptidomimetics and/or computer aided design of appropriate derivatives and analogues can be used, for example, according to the methods described above. The cell or tissue that may be employed in the method of the invention preferably is a host cell, plant cell or plant tissue of the invention described in the embodiments hereinbefore.

Determining whether a compound is capable of suppressing or activating mitogenic cyclins can be done, for example, by monitoring DNA duplication and cell division. It can further be done by monitoring the phenotypic characteristics of the cell of the invention contacted with the compounds and compare it to that of wild-type plants. In an additional embodiment, said characteristics may be compared to that of a cell contacted with a compound which is either known to be capable or incapable of suppressing or activating mitogenic cyclins.

The inhibitor or activator identified by the above-described method may prove useful as a herbicide, pesticide and/or as a plant growth regulator. Thus, in a further embodiment the invention relates to a compound obtained or identified according to the method of the invention said compound being an activator of mitogenic cyclins or an inhibitor of mitogenic cyclins.

Such useful compounds can be for example transacting factors which bind to the mitogenic cyclin of the invention. Identification of transacting factors can be carried out using standard methods in the art (see, e.g., Sambrook, supra, and Ausubel, supra). To determine whether a protein binds to the protein of the invention, standard native gel-shift analyses can be carried out. In order to identify a transacting factor which binds to the protein of the invention, the protein of the invention can be used as an affinity reagent in standard protein purification methods, or as a probe for screening an expression library. Once the transacting factor is identified, modulation of its binding to the mitogenic cyclin of the invention can be pursued, beginning with, for example, screening for inhibitors against the binding of the transacting factor to the protein of the present invention. Activation or repression of mitogenic cyclins could then be achieved in plants by applying of the transacting factor (or its inhibitor) or the gene encoding it, e.g. in a vector for transgenic plants. In addition, if the active form of the transacting factor is a dimer, dominant-negative mutants of the transacting factor could be made in order to inhibit its activity. Furthermore, upon identification of the transacting factor, further components in the pathway leading to activation (e.g. signal transduction) or repression of a gene involved in the control of cell cycle then can be identified. Modulation of the activities of these components can then be pursued, in order to develop additional drugs and methods for modulating the cell cycle in animals and plants.

The invention also relates to a diagnostic composition comprising at least one of the aforementioned nucleic acid molecules, vectors, proteins, antibodies or compounds and optionally suitable means for detection.

Said diagnostic compositions may be used for methods for detecting expression of mitogenic cyclins by detecting the presence of the corresponding mRNA which

comprises isolation of mRNA from a cell and contacting the mRNA so obtained with a probe comprising a nucleic acid probe as described above under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of the protein in the cell. Further methods of detecting the presence of a protein according to the present invention comprises immunotechniques well known in the art, for example enzyme linked immunosorbent assay. Furthermore, it is possible to use the nucleic acid molecules according to the invention as molecular markers in plant breeding.
The person skilled in the art can use proteins according to the invention from other organisms such as yeast and animals to influence cell division progression in those other organisms such as mammals or insects. In a preferred embodiment one or more DNA sequences, vectors or proteins of the invention or the above-described antibody or compound are, for instance, used to specifically interfere in the disruption of the expression levels of genes involved in G1/S transition in the cell cycle process in transformed plants, particularly :

- in the complete plant
- in selected plant organs, tissues or cell types
- under specific environmental conditions, including abiotic stress such as cold, heat, drought or salt stress or biotic stress such as pathogen attack
- during specific developmental stages.

Another aspect of the current invention is that one or more DNA sequences, vectors or proteins of the invention or the above-described antibody or compound can be used to modulate, for instance, the total architecture, growth and sensitivity to the environment of the plant concerned can be manipulated by the regulation of the expression of the cyclin gene according to the invention.

In view of the foregoing, the present invention also relates to the use of a DNA sequence, vector, protein, antibody or compound of the invention for modulating plant cell cycle, plant cell division and/or growth, for influencing the activity of cyclin-dependent protein kinase in a plant cell, for disrupting plant cell division by influencing the presence or absence or by interfering in the expression of mitogenic

cyclin for influencing cell division progression in a host as defined above or for use in a screening method for the identification of inhibitors or activators of cell cycle proteins. Beside the above described possibilities to use the nucleic acid molecules according to the invention for the genetic engineering of plants with modified characteristics and their use to identify homologous molecules, the described nucleic acid molecules may also be used for several other applications, for example, for the identification of nucleic acid molecules which encode proteins which interact with the mitogenic cyclins described above. This can be achieved by assays well known in the art such as those described above and also included, for example, as described in Scofield (Science 274 (1996), 2063-2065) by use of the so-called yeast "two-hybrid system"; see also the appended examples. In this system the protein encoded by the nucleic acid molecules according to the invention or a smaller part thereof is linked to the DNA-binding domain of the GAL4 transcription factor. A yeast strain expressing this fusion protein and comprising a lacZ reporter gene driven by an appropriate promoter, which is recognized by the GAL4 transcription factor, is transformed with a library of cDNAs which will express plant proteins or peptides thereof fused to an activation domain. Thus, if a peptide encoded by one of the cDNAs is able to interact with the fusion peptide comprising a peptide of a protein of the invention, the complex is able to direct expression of the reporter gene. In this way the nucleic acid molecules according to the invention and the encoded peptide can be used to identify peptides and proteins interacting with mitogenic cyclins. It is apparent to the person skilled in the art that this and similar systems may then further be exploited for the identification of inhibitors of the binding of the interacting proteins.

Other methods for identifying compounds which interact with the proteins according to the invention or nucleic acid molecules encoding such molecules are, for example, the in vitro screening with the phage display system as well as filter binding assays or "real time" measuring of interaction using, for example, the BIAcore apparatus (Pharmacia); see references cited supra.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present

invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The present invention is further described by reference to the following non-limiting figures and examples.

The Figures show:

Figure 1: Sequence alignment of the *A. thaliana* D-type cyclins. The CYCD4;1-coding region is aligned with CYCD1;1, CYCD2;1, CYCD3;1 (GenBank accession numbers X83369; X83370; and X83371, respectively). Alignment was obtained by the use of the PILEUP program (Genetic Computer Group, Madison, WI). Black regions indicate positions at which more than half of the sequences are identical. Shaded residues represent conserved substitutions. To obtain maximal similarity, gaps were introduced, represented by dots.

The Examples illustrate the invention:

Unless stated otherwise in the examples, all recombinant DNA techniques are performed according to protocols as described in Sambrook et al. (1989), Molecular Cloning : A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY or in Volumes 1 and 2 of Ausubel et al. (1994), Current Protocols in Molecular Biology, Current Protocols. Standard materials and methods for plant molecular work are

described in Plant Molecular Biology Labfase (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications (UK).

Example 1: Identification of a cell cycle interacting protein

To identify CDC2aAt-interacting proteins a two-hybrid system was used based upon GAL4 recognition sites to regulate the expression of both *his3* and *lacZ* reporter genes. The pGBTCDC2A vector, containing a fusion protein between the C-terminus of the GAL4 DNA-binding domain and CDC2aAt was constructed by cloning the full-lenght coding region of *CDC2aAt* into the pGBT9 vector. For the screening a GAL4 activation domain cDNA fusion library was used, constructed from mRNA of *Arabidopsis thaliana* cell suspensions harvested at various growing stages: early exponential, exponential, early stationary, and stationary phase. The pGBTCDC2A plasmid was cotransformed with the library into the HF7c reporter strain. Approximately 1.2 10^7 independent transformants were screened for their ability to grow on histidine-free medium. A 3-day incubation at 30°C yielded about 1200 colonies. These colonies were tested for their growth on medium without histidine in the presence of 10 mM 3-amino-1,2,4-triazole, reducing the number of positives to 250. Next these colonies were tested for the activation of the *lacZ* gene, and 153 turned out to be both His^+ and $LacZ^+$. DNA was prepared from the positive clones and sequenced. One of the clones contained a gene (LDV59) encoding a novel mitogenic cyclin. The LDV59 encoded protein also contains the Rb interacting motif.

The specificity of the interaction between the LDV59 encoded protein with CDC2aAt was verified by the retransformation of yeast with pGBTCDC2A and pGADLDV59. As controls, pGBTCDC2A was cotransformed with a vector containing only the GAL4 activation domain (pGAD424); and pGADLDV59 was cotransformed with a plasmid containing only the GAL4 DNA-binding domain (pGBT9). Transformants were plated on medium with or without histidine. Only transformants containing both pGBTCDC2A and pGADLDV59 were able to grow in the absence of histidine.

Example 2: The LDV59 encoded protein associates with both Cdc2aAt and Cdc2bAt

The pGBTCDC2B vector encoding a fusion protein between the C-terminus of the GAL4 DNA-binding domain and CDC2bAt was constructed by cloning the full-lenght coding region of *CDC2bAt* into the pGBT9 vector. pGBTCDC2B was transformed with pGADLDV59 in the HF7c yeast and cotransformants were plated on medium with or without histidine. As controls, pGBTCDC2B was cotransformed with a vector containing only the GAL4 activation domain (pGAD424); and pGADLDV59 was cotransformed with a plasmid containing only the GAL4 DNA-binding domain (pGBT9). Only transformants containing both pGBTCDC2B and pGADLDV59 were able to grow in the absence of histidine. These data demonstrate that the LDV59 encoded gene product not only binds to CDC2aAt, but also to CDC2bAt.

Example 3: Characterization of the LDV59 gene

To study the genomic organization of the LDV59 gene, *Arabidopsis* DNA was digested with 3 different enzymes. Hybridization with the LDV59 coding region at low stringency showed only one band for every digest, indicating the presence of only one LDV59 gene per haploid genome of *Arabidopsis*.

Example 4: LDV59 expression is mitogenic inducible

Arabidopsis cell suspensions ecotype Col.-O, maintained as described by Glab et al. (FEBS Lett. 353 (1994), pag. 207-211) were depleted for growth factors for 48 hrs. by resuspending them in medium lacking auxin (2,4-D), cytokinin (BAP), and sucrose. After 48 hrs. the cells were split into eight aliquots, which were resuspended in medium containing sucrose or lacking sucrose, containing auxin or lacking auxin, and containing cytokinin or lacking cytokinin. After 6 hrs. cultivation RNA was extracted from the cells. An RNA gel blot was probed with the LDV59 gene. A hybridization signal was only observed for the cells supplemented with cytokinin and sucrose, indication that the LDV59 gene is specially induced by these mitogenic agents.

Example 5: LDV 59 expression

Plant material was fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH7.2). Fixed tissue was dehydrated with ethanol, cleared with toluene, and embedded in paraffin. Embedded tissue was sliced into serial 10μm sections and attached to coated microscope slides. ^{35}S-UTP-labeled sense and antisense RNA of a cDNA LDV59 subcloned in PGem2 were generated by run-off transcription using T7 and Sp6 RNA polymerases according to the manufacturer's instructions (Boehringer Mannheim). Full-length transcripts were reduced to an average length of 0.3kb by alkaline hydrolysis (Cox et al., 1984, Dev.Biol.,101, p.485). The size of both full-length and hydrolyzed transcripts was checked on a 1% denaturing gel, and the amount of synthesized RNA was calculated. The mRNA in situ hybridization procedure was carried out essentially as described by Angerer and Angerer (1992) in "In situ hybridization": A practical approach (The Practical Approach series, Wilkinson DG,ed,Oxford Press,pp.15-32). Stringent wash was performed in 0.1SSC (1X SSC; 150mM NaCl, 15mM Na3-citrate, pH7.0) at 62°C for 60 min to avoid cross hybridization.

By applying the mRNA *in situ* hybridization technique on roots, shoot apical meristems and flowers of Arabidopsis thaliana and on radish roots, the following expression patterns were observed.

Early, during lateral root development, pericycle cells neighbouring one protoxylem pole show an intense hybridization signal. As lateral roots expand very strong signal is mainly observed at its basis. At that stage a weak or no signal is detected in the meristematic cells of the lateral root tip. At later stages of root development, only a subset or group of cells in the root meristem show high accumulation of LDV59 mRNA. Mature root meristems barely show any expression of the LDV59 gene. Cell files in specific regions along the vascular tissue show a weak and uniform expression pattern. Similar cell files of the vascular tissue show a completely different pattern of expression. Alternating stretches of cells along the vascular cylinder expressing and not expressing the LDV59 are observed. Some tissue sections along the vascular tissue contained only one single cell expressing the LDV59 gene.

During flower development LDv59 expression was observed along the vascular tissue from the filament and young ovaries. At later stages, high accumulation of LDV59 mRNA was observed in the fertilized ovule, most likely in the megaspore-mother-cell burried in the nucellus tissue. During embryo development expression was high in globular and heart stages embryos and low in mature embryo. These results shows that LDV59 is involved in early steps of vascular tissue, lateral root formation and embryo development.